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Regulation of the Nuclear Factor of Activated T Cells in Stably Transfected Jurkat Cell Clones

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Two Jurkat cell clones have been stably transfected with a reporter vector for the nuclear factor of activated T cells (NFAT). Upon stimulation, they express high levels of secreted heat stable placental alkaline phosphatase. With these clones, we demonstrated that NFAT activation induced by phorbol 12-myristate 13-acetate and ionomycin was inhibited by both cyclosporin A (CsA) (IC_{50} = 8 nM) and FK506 (IC_{50} = 160 pM), presumably by inhibition of calcineurin activity. Selective phosphatase inhibitors for protein phosphatase 1 (PP1) and 2A (PP2A) that do not inhibit calcineurin, such as okadaic acid and calyculin A, also inhibited NFAT activation with IC_{50}s of 87 nM and 4 nM, respectively, suggesting that okadaic acid and related inhibitors may block NFAT activation through the inhibition of PP1, instead of PP2A. NFAT activation was also inhibited by agents that increase cAMP concentrations such as dibutyryl cAMP, forskolin and prostaglandin E_2. These stable Jurkat cell clones provide a convenient and sensitive tool to study NFAT regulation.

The nuclear factor of activated T cells (NFAT) is a transcriptional factor that regulates cyclosporin (CsA) and FK506-sensitive transcription of cytokine genes (1). Two different NFATs have been identified and cloned (2, 3). $NFAT_p$ is a 120 kDa pre-existing cytosolic factor in most of lymphoid lineage cells, and $NFAT_c$ is a 78 kDa inducible protein selectively expressed in activated T cells. $NFAT_c$ shares about 75% sequence identity with $NFAT_p$ in the DNA-binding domain and about 3% identity in the C-terminal region (1-3). Upon stimulation, NFATs translocate into the nucleus to form a complex with Fos-Jun and Jun-Jun dimers (AP-1) and bind to the distal NFAT site of the interleukin-2 (IL-2) promoter (4, 5). The immunosuppressive drugs CsA and FK506 are potent inhibitors of the activation of NFATs (6). When these drugs bind to their cognate binding proteins, cyclophilins and FK506-binding proteins (FKBPs), the drug-immunophilin complex inhibits calcineurin (CaN), a Ca^{++}/calmodulin-dependent serine/threonine phosphatase (protein phosphatase 2B, PP2B) that is essential for the translocation and activation of NFATs (4, 7, 8). In this study, we have stably transfected selected two Jurkat cell clones with a reporter vector for NFAT. Using these clones, we have identify several potential factors that may modulate NFAT activation.

MATERIALS AND METHODS

Transfection and clone selection. Jurkat cell lines with stable integrants of the constructs were established by electroporating a linearized NFAT reporter plasmid (SX-NFAT) (9), a generous gift from Dr. G.R. Crabtree, into Jurkat cells and selecting with G418. Briefly, the plasmid has three repeated NFAT binding sequences in the promoter region followed by a reporter protein, heat stable secretory placental alkaline phosphatase (SEAP) (10). The cells were maintained in complete RPMI 1640 (Gibco) supplemented with 10% v/v fetal calf serum, 100 U/ml penicillin and 50 U/ml streptomycin in 5% CO_2. Three million cells were electroporated with 20 μg of plasmid, previously linearized with Pvu l, in 1 ml RPMI 1640 using a 250 V and 960 μF electric shock (Bio-Rad). Immediately after electroporation, cells were suspended in 10 ml of tissue culture media. G418 (1 mg/ml) was added to the culture media after 48 hrs. G418-containing media was changed periodically and resistant cells grew out 4 weeks later. G418-resistant cells were sorted into a 96-well plated at one cell per well using a flow cytometer (Becton-Dickinson) and cultured in the presence of G418 (1 mg/ml) for 3 weeks with periodic media changes. Clones that expressed the most SEAP after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin were selected.

Stimulation of cells. Exponentially growing cells at less than 0.7×10^6 cells/ml were stimulated with PMA (50 ng/ml)

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and ionomycin (1 μM) for 20 h in fresh tissue culture media in a 96-well plate at a density of 0.5×10^6 cells/ml in the presence or absence of the indicated agents.

SEAP activity assay. SEAP levels were determined as described previously (10). Briefly, media were removed from each well, heated at 65° for 60 min to inactivate intrinsic heat-sensitive alkaline phosphatase and centrifuged at 10,000 × g for 2 min. An aliquot of media (100 μl) was mixed with 100 μl of SEAP assay buffer (1.0 M diethanolamine pH 9.8, 0.5 mM $MgCl_2$ and 10 mM L-homoarginine) in a 96-well plate. p-nitrophenylphosphate (120 mM, 20 μl) in SEAP assay buffer was added to each well and mixed. The absorbance (A_{410}) of the reaction mixture was determined at 30-min intervals. Data are presented as the percentage of the maximum linear reaction rate determined and analyzed with Student's t-test.

RESULTS AND DISCUSSION

Sixteen G418-resistant clones were studied and two clones that expressed high levels of SEAP upon stimulation with PMA (50 ng/ml) and ionomycin (1 μM) were selected and designated as NFAT-A and NFAT-B clones (Fig. 1). Both were cultured in the presence and absence of G418 for 10 weeks (13 passages) and no difference was detected in their responses to stimulation, suggesting the reporter vector was stably integrated into the Jurkat cell chromosome (Fig. 2). Although another group has selected Jurkat cell clones stably transfected with the *lac*Z reporter vector for NFAT (11), our clones provide a more convenient and sensitive tool to study NFAT.

The effect of the immunosuppressive drugs CsA and FK506 on NFAT transcriptional activity was examined on the NFAT-A clone. The activity induced by stimulation was inhibited by both CsA and FK506 in a dose dependent manner with the IC_{50}s of 8 nM and 160 pM respectively (Fig. 3).

To determine if other serine/threonine protein phosphatases are involved in NFAT activation, we also examined the effect of okadaic acid and calyculin A, which are selective inhibitors for protein phosphatase 1 and 2A (12). Both okadaic acid (IC_{50} = 87 nM) and calyculin A (IC_{50} = 4 nM) inhibited NFAT activity in a dose-dependent manner (Fig. 3). It has been previously reported that the IC_{50} for the isolated catalytic subunit of protein phosphatase-1 (PP1) from rabbit skeletal muscle was 3.4 nM for okadaic acid and 0.3 nM for calyculin A, and that the IC_{50} for protein phosphatase-2A (PP2A) was 0.07 nM for okadaic acid and 0.13 nM for calyculin A (13). From the relative sensitivity of these isolated enzymes, one might conclude that okadaic acid inhibits NFAT activity through inhibition of PP1, instead of PP2A. The greater concentration of phosphatase inhibitors required in our study undoubtedly reflects the need to achieve a rate limiting condition in whole cells as compared to inhibition of purified enzymes (14). Okadaic acid is a very weak inhibitor of calcineurin (IC_{50} = 5 μM) at concentrations that completely inhibit PP1 and PP2A

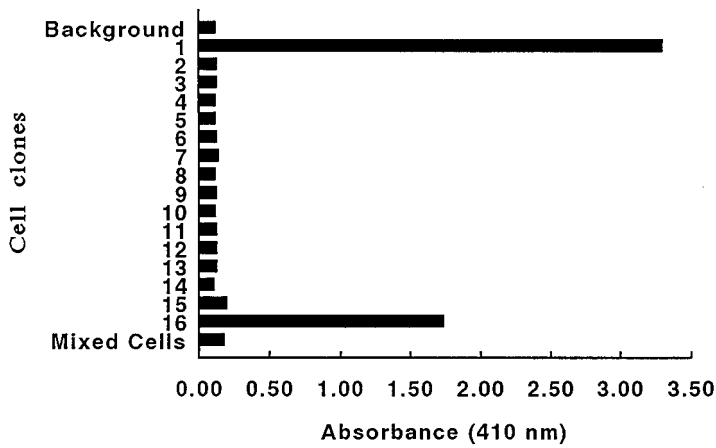

FIG. 1. Clone selection. Clones were stimulated with PMA (50 ng/ml) and ionomycin (1 μM); SEAP activity was assayed as described in Materials and Methods. Mixed Cells, heterogeneous G418-resistant cell population; Background, untransfected Jurkat cells.

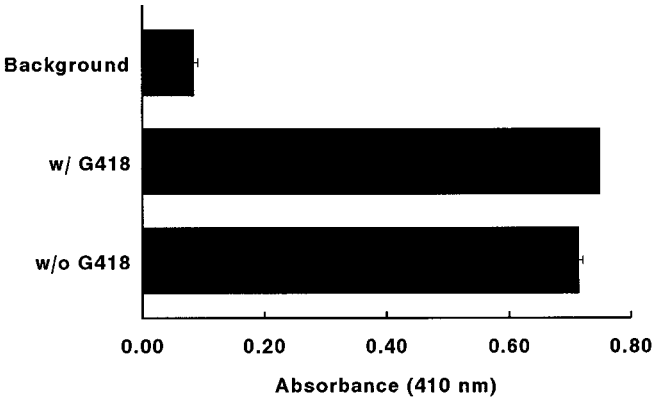

FIG. 2. Stability of the NFAT-A cell clone. After being cultured in the presence and absence of G418 for 10 weeks, NFAT-A cells were stimulated and SEAP activity determined as described in Materials and Methods. Bars, ±SD; Background, untransfected Jurkat cells.

activity (14). Okadaic acid and calyculin A did not interfere with the SEAP analysis (data not shown). As a control, we constructed an eukaryotic expression vector (pCEP4/SEAP) which expresses SEAP under the control of the cytomegalovirus (CMV) promoter. Okadaic acid and calyculin A did not inhibit the synthesis and secretion of SEAP in this cell line when stimulated with PMA and ionomycin (data not shown).

Many protein kinases are involved in the signaling pathway of T cell activation. cAMP-dependent protein kinase (PKA), a well-characterized serine/threonine protein kinase, negatively regulates the IL-2 expression (15). The possible regulatory role of PKA in NFAT activity was also investigated with the NFAT-A clone. Our results demonstrate that dibutyrul-cAMP (1 mM), but not dibutyryl-cGMP (1 mM), inhibits nearly 90% of NFAT activity (Fig. 4). Forskolin (10 μM), an adenylate cyclase stimulator, also inhibited NFAT activity by 75%. Prostaglandin E_2 (PGE_2) (0.1 μM), which significantly increases cytosolic cAMP concentration, also inhibited NFAT activity by 48%, suggesting that PGE_2 inhibition of IL-2 expression (16) is in part through the inhibition of NFAT function. Although the specific mechanism of PKA inhibition of NFAT activity is unknown, we suggest that PKA may phosphorylate a serine/threonine site(s) on $NFAT_p$ and block its trans-

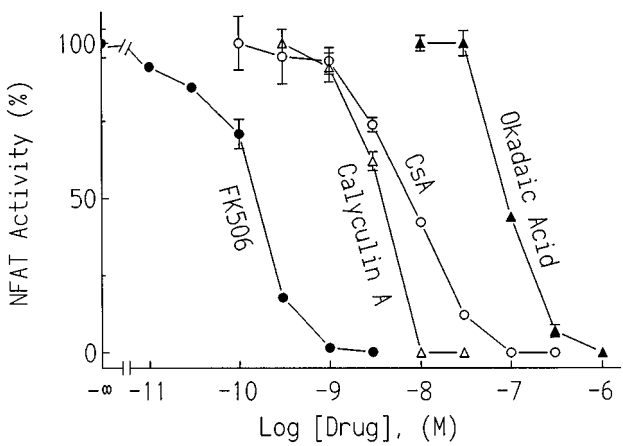

FIG. 3. NFAT activation in NFAT-A cells is inhibited by CsA, FK506, okadaic acid or calyculin A. Cells were stimulated with PMA and ionomycin in the presence of different concentrations of CsA, FK506, okadaic acid or calyculin A. SEAP activity was determined as described in Materials and Methods. Bars, ±SD.

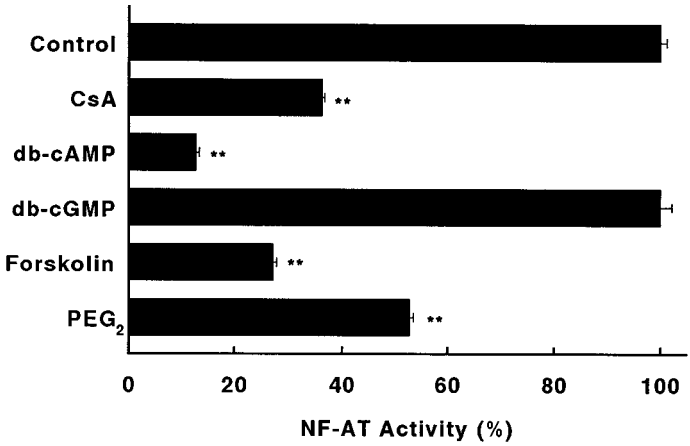

FIG. 4. NFAT activation is inhibited by dibutyryl-cAMP, forskolin and PGE_2. Cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) in the presence of CsA (10 nM), dibutyryl-cAMP (1 mM), dibutyryl-cGMP (1 mM), forskolin (10 μM) or PGE_2 (0.1 μM). SEAP activity was determined as described in Materials and Methods. Control, stimulation only; Bars, ±SD; **, $P < 0.01$.

location and activation. It is also possible that PKA inhibits NFAT activity indirectly by modulating the calcineurin- or okadaic acid-sensitive mechanism.

In summary, we have transfected Jurkat cell with a NFAT reporter vector and selected two stable clones that provide a convenient and efficient tool to study the control of NFAT activation in T cell.

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